Amino Acids (2014) 46:153–165
DOI 10.1007/s00726-013-1614-x

INVITED REVIEW

Protein renaturation with simultaneous purification by protein folding liquid chromatography: recent developments

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Received: 25 July 2013 / Accepted: 20 October 2013 / Published online: 14 November 2013

Abstract Protein folding liquid chromatography (PFLC) is a powerful tool for protein refolding with simultaneous purification. We review its recent progress in liquid chromatography and molecular biology, primarily involving the validation of PFLC refolding of proteins containing multiple disulphide bonds, the application of mixed-mode chromatography, PFLC in molecular biology. Representative examples are described.

Keywords Protein folding liquid chromatography (PFLC) · Proteins · Inclusion bodies · Refolding with simultaneous purification · Mixed-mode liquid chromatography

Abbreviations

PFLC	Protein folding liquid chromatography
LC	Liquid chromatography
MMC	Mixed-mode chromatography
E. coli	*Escherichia coli*
N state	Native state
U state	Unfold state
M state	Intermediates state
HIC	Hydrophobic interaction chromatography
IEC	Ion exchange chromatography
SEC	Size exclusion chromatography
AFC	Affinity chromatography
RPLC	Reverse phase liquid chromatography
EBA	Expanded bed chromatography
SMB	Simulated moving bed
PFC	Perfusion chromatography
USRPP	The units of simultaneous renaturation and purification of proteins
AMC	Artificial molecular chaperone
AMC-IEC	AMC-ion exchange chromatography
GdnHCl	Guanidine hydrochloride
DTT	Dithiothreitol
β-ME	β-Mercaptoethanol
HI-EBAC	Hydrophobic interaction expanded bed adsorption chromatography
INAC	Immobilised nickel affinity chromatography
D-LC-1C	A single column
2D column	Two-dimensional column
2D-LC-1C	2D with a single column
WCX	Weak cation exchange
WAX	Weak anion-exchange chromatography
STHIC	Stationary phase of hydrophobic interaction chromatography
rhG-CSF	Recombinant human granulocyte colony stimulating factor
NCp-NiV	Nipah virus
HBx	Hepatitis B virus X
rhIFN-γ	Recombinant human interferon-γ
rhEGF	Recombinant human EGF
rhSCF	Recombinant human stem cell factor
rhAFP	Recombinant alpha-fetoprotein
rhFL	Recombinant human Flt3 ligand
rhIFN-alpha-2b	Recombinant human interferon-alpha 2b
LK68	Lipoprotein kringle
rhVEGF	Recombinant human vascular endothelial growth factor

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rhDll1	Recombinant human Notch ligand delta-like 1
GSH/GSSG	Glutathione/glutathione
α-Chy	α-Chymotrypsin
Cyt C	Cytochrome C
MOP	Molecular orientation of protein
SDT	Stoichiometric displacement theory
PEG	Polyethylene glycol
CBD	Cellulose-binding domain
PPI	Peptidyl prolyl cis trans isomerase
PDI	Protein disulphide isomerase
α-Amy	α-Amylase
LC–MS	LC–mass spectrometry

Introduction

Protein folding liquid chromatography (PFLC) has been defined as "a kind of liquid chromatography, incorporating various kinds of biochemical and/or physicochemical processes originally accomplished in solution, which can result in either raising the efficiency or shortening the time of protein folding" (Geng and Wang 2007). In the 20 years since the development of in vitro protein refolding by PFLC, this approach has become not only an effective protein refolding method in biological chemistry, but also a branch of liquid chromatography (LC) (Geng and Wang 2008). PFLC has developed so rapidly that based on recent statistics from the internet (http://www.ScienceDirect.com), this approach has been reported more than 12,800 times. In the past year, these records have increased more than 1,300 times. Many review articles and books have been published to introduce this novel technology (Phadtare et al. 1994; Schlegl et al. 2003; Swietnicki 2006; Park et al. 2006; Rinas et al. 2007; Geng and Wang 2007, 2008; Lu et al. 2010; Li and Leong 2011; Bai and Geng 2011). Including all of the developments in this article is challenging due to the diversity in the applications of this approach. We primarily address PFLC validation, the refolding of proteins containing multiple disulphide bonds, and the application of mixed-mode chromatography (MMC). Representative examples are described.

Recombinant protein expression in *Escherichia coli* (*E. coli*) has thus far been the primary means of producing large numbers of medicinally relevant proteins and other proteins of research interest (Baneyx and Mujacic 2004; Vermasvuori et al. 2009). Three main methods have been employed to express eukaryotic genes in *E. coli*, including insoluble intracellular expression, soluble intracellular expression, and secretion expression. Foreign gene is often located in the cytoplasm, periplasmic space, inner or outer membrane and matrix outside cells. In the past, insoluble intracellular expression is the main way, accounting for more than 80 %. One problem that is sometimes encountered is that proteins are valuable for research or biomedicine and proteins expressed in this way exist as insoluble inclusion bodies (Cabrita and Bottomley 2004). The refolding of these inclusion bodies is a 'bottleneck' for obtaining large quantities of active proteins (Špela and Radovan 2011).

As the understanding of the genetic characteristics of *E. coli* has solidified, *E. coli* has emerged as a source for desired functional proteins using recombinant methods. The production of heterologous proteins in *E. coli* at very high levels overcomes the problematically low natural expression levels of many proteins, which limits protein research and applications. For proteins that require genetic engineering and a high output of therapeutic protein in industry, understanding active refolded protein conformations and improving the refolding efficiency of inclusion bodies are key to successfully obtaining active target proteins in vitro. Various protein refolding technologies have been developed to solve these problems (Geng and Wang 2007, 2008; Raghava et al. 2008; Li and Leong 2011).

In PFLC, protein folding processes are at the interface between liquid (mobile phases) and solid (stationary phases) (Geng and Wang 2007, 2008). Protein in unfold (U) state cannot only obtain high enough free energy at the solid–liquid interface, but also raise up refolded efficiency by many times of the adsorption–desorption of the target proteins. In short, the interface and the association between stationary and mobile phases play a cardinal role. Thus, the interfaces of hydrophobic interaction chromatography (HIC), ion exchange chromatography (IEC), and affinity chromatography (AFC) have been used to refold and purify proteins; these approaches are simply expressed as PF-HIC, PF-IEC and PF-AFC, respectively. Protein in U state can also be refolded by the forth kind PFLC, size exclusion chromatography (SEC, also expressed with PF-SEC as protein folding). However, protein does not interact with stationary phase, its separation with simultaneous refolding processes due to various sizes of protein in U and N state. Although the refolding efficiencies vary between different proteins, all proteins fold more efficiently using this method than using buffer methods (Wang et al. 2008a, b; Luo et al. 2011).

Semi-preparative and preparative PFLC methods such as expanded bed chromatography (EBA) (Sharma et al. 2008), simulated moving bed (Gueorguieva et al. 2011), perfusion chromatography (Gorka et al. 2012), chromatographic cake or the unit of simultaneous renaturation and purification of proteins (USRPP) have emerged to allow purification scale-up (Geng and Zhang 2008). Specifically, the recent appearance of MMC has provided more flexible conditions for optimisation by PFLC.

Protein renaturation with simultaneous purification by PFLC

Model proteins

In some cases, new polypeptide chains can easily fold into their corresponding stable N states with active natural conformations that are consistent with their amino acid sequences. Therefore, the standard protein chosen as a model to study renaturation in vitro should have certain advantages for examining the refolding of denatured and refolded proteins, such as a stable structural conformation and biological activity (Swaminathan et al. 2011).

Model proteins are dissolved in high concentrations of denaturing agents, such as 8 M urea or 6–7 M guanidine hydrochloride (GdnHCl) solution, and the denatured model proteins are directly injected onto the chromatographic column for protein refolding and simultaneous purification. Lysozyme was very encouraging as a model protein for investigating protein folding by PFLC. A new method of artificial molecular chaperone-IEC has been applied for the refolding of urea-denatured/dithiothreitol-reduced lysozyme. At a high initial protein concentration of 200 mg/mL, the recovered activity was greater than 60 % (Wang et al. 2010a).

The enzyme α-chymotrypsin (α-Chy) is another model protein for the investigation of protein folding. This enzyme is auto-catalytic, even in its solid state. The auto-catalytic products including peptides, unfolded intermediates, or even aggregates that always coexist. Therefore, this system is favourable for studying protein folding under an equilibrium condition among α-Chy in the N state, peptides, unfolded intermediates, and aggregates. Using urea-denatured α-Chy, the refolding efficiencies of two types of HIC stationary phases were investigated, providing further evidence that the stationary phase plays a key role in protein refolding (Liu et al. 2009b). An approach for refolding denatured proteins in proteome research by HIC-PFLC has recently been reported. The equilibrium among the U state, folded intermediates (M state) and N state was investigated during protein folding under various denaturant concentrations (Ke et al. 2013). The specific bioactivity of the refolded α-Chy was found to be higher than that of commercial α-Chy as the urea concentration in the sample solution varied between 1.0 and 3.0 M; the highest specific bioactivity occurred at the urea concentration of 1.0 M, indicating the possibility of refolding of some proteins that have partially or completely lost their bioactivity due to the use of a dilute urea solution to dissolve the sample. When the urea concentration reached 6.0 M, the unfolded α-Chy could not be refolded at all.

Renaturation with simultaneous purification of inclusion bodies by PFLC

Inclusion bodies are insoluble in aqueous solutions; therefore, dissolving solutions must be utilised and then removed. Two steps are involved in the process of inclusion body refolding. First, inclusion bodies are dissolved in a denaturant solution with a high concentration of urea or GdnHCl and a small amount of reducing agent, such as dithiothreitol or β-mercaptoethanol, to break disulphide bonds. The dissolved proteins exist as elongated polypeptide chains without biological activity. Second, the stretched polypeptide chains refold when the denaturant is removed. However, many incorrect 3D structures persist throughout this process, and some precipitation can occur (Carl 2007).

The direct recovery of recombinant nucleocapsid protein from the Nipah virus from crude *E. coli* homogenate was successfully developed using a hydrophobic interaction expanded bed adsorption chromatography (Chong et al. 2010). New advances have increased protein mass recovery using PFLC.

Several representative examples from the literature between 2008 and 2013 of new applications for protein folding using PF-HIC, PF-SEC, PF-IEC, and PF-AFC (alone or in combination) are listed in Table 1. The most important parameters for evaluating the efficiency of protein refolding, such as mass recovery, specific bioactivity, and production costs in large-scale production, were tabulated. Regardless of the type of PFLC that was selected, the purity of all listed refolded and simultaneously purified proteins was >95 %. This level of purity meets the requirements of therapeutic or diagnostic proteins.

Notably, the characteristics of the PF-AFC method shown in Table 1 have prompted scientists to construct a number of recombinant proteins as affinity tags. This development led to the first chromatography refolding-based bioprocess for hepatitis B virus X protein (HBx) using immobilised nickel affinity chromatography (Anindya and Leong 2012). The authors used a statistical design for the experimental methodology to create an optimum process that delivered bioactive HBx at 0.21 mg/mL/h with a refolding yield of 54 %, which is 4.4-fold higher than that, achieved using dilution refolding. This process enabled the production of HBx at quantities and purities that facilitates its direct use in structural and molecular characterisation studies. However, the incubation of the reduced-denatured protein on the employed column (which entails five column volumes) requires at least 1 day. Of course, the use of AFC will increase costs, posing a challenge for industrial production (Waugh 2005).

Regardless of which of the four methods listed in Table 1 is employed, a common problem is that denatured

Table 1 Typical examples of PFLC for refolding with simultaneous purification of recombinant proteins

FPLC types	Proteins	Types of stationary phase	Purity (%)	Mass recovery or yield (%)	A specific bioactivity (IU/mg)	References
AFC	rAFP-C	IMAC	98	60	NR	Sharapova et al. (2011)
	rhBMP-2	Ni-NTA	NR	97	NR	Zhang et al. (2011)
	Lipase	Ni-NTA	NR	NR	3,000	Akbari et al. (2010)
	scFv	Ni-NTA	96	44	NR	Wang et al. (2010b)
	rhG-CSF	IMAC	97	39	2.3×10^8	Wang et al. (2009a)
	TRAIL	Ni-NTA	98	NR	NR	Wang and Shi (2009)
IEC	Tα1-TP5	Superdex 30 prep grade	95	NR	NR	Li et al. (2012a, b)
	α-Fetoprotein	Q-FF	N.R	85	N.R	Chen and Leong (2009)
		DEAE-FF	N.R	81	N.R	
	rhSCF	SAX (-SO_3H)	96.3	43	7.8×10^5	Wang et al. (2008b)
	rhEGF	Toyopearl-SP	>99	NR	5.0×10^5	Sharma et al. (2008)
	β_2-M	SAX	NR	NR	NR	Bertoietti et al. (2013)
HIC	rhFlt3	PEG400	94.5	36.9	N.R.	Jia et al. (2010)
	rhSCF	PEG200-800, THFA	95.5	49.6	1.26×10^6	Wang et al. (2011b)
	rhIFN-γ	Octyl-Sepharose	99	NR	2×10^8	Petrov et al. (2010)
		PEG200	95	92	9.5×10^8	Wu et al. (2008)
		PEG600	95	NR	8.7×10^7	Bai and Geng (2011)
	rhG-CSF	PEG600	95.4	36.9	2.3×10^8	Wang and Geng (2012)
SEC	DT389-hIL13	Superdex-75/G-25	95	NR	NR	Sun et al. (2011)
	TAT-Nrf2	Superose 6 10/300	99	36	NR	Robert and Richard (2010)
	AFP	Superdex 200 10/300	46	60	NR	Chen and Leong (2010)
	hApaf-1	Sephadex G100	100	NR	NR	Nageswara et al. (2009)
SEC/IEC	CBS	G-25/DEAE-FF	NR	NR	NR	Majtan and Kraus (2012)
IEC/SEC	MTI 2	Mono Q/Superose 12	NR	50	NR	Alessandra et al. (2009)
	Streptokinase	DEAE 650 M/Superdex 75	99	40 mg	1.5×10^5	Cherish et al. (2008)
AFC/IEC	AcpH	Ni–NTA/Superdex 200/Superdex 75	93	51.2	NR	Murugan et al. (2010)
AFC/SEC	Anti-CD25	Ni–NTA/Superdex 75	95	15	RA 91 %	Wang et al. (2008c)
AFC/SEC	rhDelta-like1	GSTrap AFC/Sup erdex-75	99	40	NR	Shi et al. (2008)

NR not reported, *RA* relative activity

proteins that are solubilised in solution (7.0 M GdnHCl or 8.0 M urea) will form precipitates upon injection onto the column and contact with the aqueous solution. Protein refolding can be initiated from only monomer denatured states; therefore, precipitation will not only affect the mass and activity recoveries, but also block frits on the top of columns during sampling. Although USRPP or chromatographic cake, which has a much greater diameter than thickness, can be used for protein refolding and simultaneous purification (even in the case of precipitate formation), these deposits should be regularly cleaned. High concentrations of denaturing agent solution have been used to clean the columns at a very low flow rate. The collected fraction was re-injected into the column to recover the rhIFN-γ, but the process was slow (Wu et al. 2007).

Employing an on-line mixed-mode column or on-line two-dimensional liquid chromatography using a single-column (2D-LC-1C) device allows cleaning and recovery of the target protein (Fig. 1) (Geng et al. 2009). The device can collect the dissolved fraction of protein in the U state in the sample collector or sample loop group (each loop is 5 or 10 mL) of the device in an on-line manner. The cleaning operation can be conducted at extremely low flow rates at night and can be stored in the sample box of the device at 4 °C, all in a closed system according to the same procedure as on-line protein separation by 2D-LC-lC.

The aforementioned new technologies have been employed in large scales. "EBA was used to perform large-scale refolding with simultaneous purification of the recombinant lipoprotein kringle (Choi et al. 2005)." When EBA-ion exchange chromatography was employed to refold recombinant human EGF (rhEGF), up to 99 % purity and 5×10^5 IU/mg specific activity were observed, and the SEC profile showed that minimal aggregates were present, with 99 % renatured active protein (Sharma et al. 2008). Chromatographic cake or caky column has been

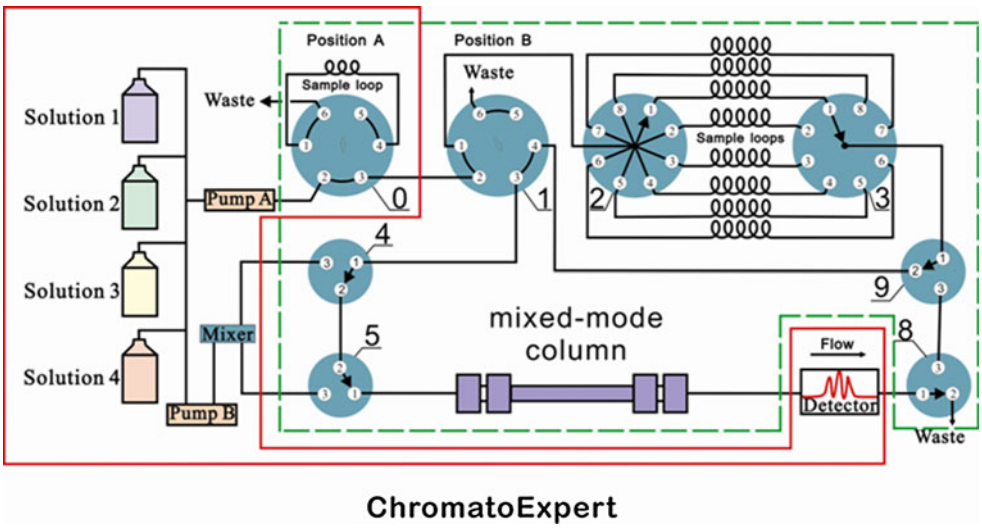

Fig. 1 Installation of on-line single-column two-dimensional liquid chromatography

used in the refolding with simultaneous purification of recombinant human stem cell factor (rhSCF) (Wang et al. 2011b). Using a chromatographic cake with a thickness of 1.0 cm and diameter of 5.0 cm, 2 mL of extract in 8.0 M urea solution was directly and continuously injected by pumping onto the chromatographic cake. The purity of the final rhSCF was greater than 95.8 %, and the specific bioactivity was greater than 2×10^6 IU/mg after only one step of PF-HIC over 40 min. Further, PF-HPHIC was applied to the refolding of rhG-CSF, which was solubilised by 8.0 M urea from the inclusion bodies, then processed on a laboratory scale (10 mm × 20 mm i.d.) and large scale (10 mm × 200 mm i.d.). Two hundred millilitres of rhG-CSF solution solubilised by 8.0 M urea (containing a total of approximately 1.6 g of protein) could be loaded onto the large column at one time. Under these conditions, the obtained rhG-CSF had a specific activity of 2.3×10^8 IU/mg and purity of 95.4 %, and the mass recovery during the purification was 36.9 % (Wang and Geng 2012). Compared with the early work using rhIFN-γ (Geng et al. 2006) on large scales (in which the flow rate was 120 mL/min and required 40 min for a chromatographic run), the flow rate here was only 2.0 mL/min and required 140 min for the necessary purification and renaturation. Both the low mass recovery and long running time seem to limit the practical production of rhG-CSF by PFLC. However, from the standpoint of drug economics, the high ratio of loading/bed volume here (1.6 g of proteins/0.31 4 L or 5 mg/mL) is high enough. Compared with conventional dilution and dialysis methods, this approach still has some advantages.

Various types of silica-based stationary phase and polymer supports, such as metal chelate columns based on cross-linked poly glycidyl methacrylate continuous rods and packings based on glycidyl methacrylate-butyl methacrylate-adipic dimethacrylate copolymer, have been employed for protein refolding with purification (Wang and Shi 2009). For ligands bound to a support, artificial chaperones use a variety of assisting agents, including polyethylene glycol (PEG), cyclodextrin, arginine, proline, surfactants, and detergents; these chaperones have been an intensely researched topic (Li et al. 2009; Antonio-Perez and Ortega-Lopez 2010). Hil'chuk et al. (2006) have prepared a stationary phase immobilised with an IFN-alpha-2b single-chain antibody and cellulose-binding domain fusion protein for the refolding and purification of IFN-alpha2b with very good results.

The recovery of mass and bioactivity by PFLC are the two main factors to evaluate the efficiency of protein refolding, but mass recovery is of primary importance. Mass loss originates from sampling and irreversible adsorption during chromatography. The features of N state proteins are well-known, but U states and stable M states of protein unfolding and/or refolding are less understood (Radford et al. 1992).

Refolding with simultaneous purification of proteins containing multi-disulphide bonds

Many difficulties still exist in the refolding of inclusion bodies that contain many disulphide bonds on FPLC columns, such as their complicated nature (95 % of the eukaryotic proteins contain disulphide bonds), low refolding efficiency, low activity recovery and low mass recovery (low correct matching rate of disulphide bonds). For a protein containing four pairs of disulphide bonds, 105 possible pairings exist. Accurately achieving protein renaturation or refolding either in solution or on the FPLC column is difficult.

Greater numbers of disulphide bonds lead to increased difficulty in protein refolding. Several groups have made significant progress in resolving this problem. A simple

anion-exchange column was employed to successfully refold in a single step with simultaneous purification of semi-crude rhAFP with 16 disulphide bonds (Chen and Leong 2009). After only 3 h, rhAFP was refolded with a yield of 28 % and product purity of 95 % at a 1 mg/mL protein refolding concentration. Compared with dilution refolding, the on-column rhAFP refolding productivity was ninefold higher, while that of off-column refolding was more than an order of magnitude higher. This work extends the application of PFLC to a myriad of complex industrial inclusion bodies. The authors expected that this method will be employed on large scales, but the approach still requires 3 h of protein incubation on the column to accomplish the redox reaction to correctly form disulphide bonds.

Due to kinetic problems in the refolding of proteins containing many disulphides, many investigations have reported off-column protein refolding in a container as a combination on-line-off-line method (Bi et al. 2010; Lu and Chang 2010; Wang et al. 2011a). A typical off-line example employs smart polymers to interact with and/or adsorb unfolded protein under certain conditions and subsequently form polymer-protein complex precipitates under other conditions (Gautam et al. 2012). After separating the liquid and solid phases with simultaneous separation from impure proteins, the bonded proteins are desorbed by a suitable solution, again with simultaneous separation from other impure proteins. The target proteins may refold during adsorption, desorption, or both. The refolding of eight different proteins was performed by precipitation with smart polymers. These proteins have different molecular masses and different numbers of disulphide bonds, and some are highly prone to aggregation (Ito et al. 2012). These proteins can be dissociated and recovered after the refolding step. The refolding can be scaled-up, with high refolding yields in 8 M urea solution (for CD4D12, the first two domains of human CD4) of up to 58 mg/L (for malETrx). The time for maximum binding was approximately 50 min, which coincided with the time required for incubation with the polymer that gave the maximum recovery of folded proteins. Biological activity assays for thioredoxin and a fluorescence-based assay, in the case of maltose binding protein, were also performed to confirm correct refolding. A novel LC column was reported for the renaturation of reduced-denatured ribonuclease with PF-IEC by bonding glutathione/glutathione (GSH/GSSG) to a silica-based stationary phase (Ke and Meng 2012). This bonded phase not only has redox reaction character, which favourably forms the correct four disulphide bonds, but also performs weak cation exchange (and actually also has HIC mode, see "MMC" later) to achieve conventional protein refolding by PFLC. The results are listed in Table 2.

As shown in Table 2, although the bonded GSH/GSSG stationary phase can significantly increase refolding efficiency, the dynamic factor still limits the PFLC speed. Therefore, exploring catalysis methods to accelerate the correct coupling of disulphide bonds is necessary. The "future" section of the previous review noted that "many difficult problems are encountered during the folding of a denatured/reduced protein, including both thermodynamic problems (correct forming of many disulphide bonds existing in a protein molecule) and kinetic problems (rapid forming of disulphide bonds)" (Geng and Wang 2007). This problem has not yet been solved.

One question related to the use of PFLC is that which inclusion bodies can be refolded with simultaneous purification using this approach. In theory, any type of denatured protein can be refolded with simultaneous purification by any of the aforementioned four types of PFLC. However, from a practical standpoint, it is prudent to determine which of the four PFLC types is best.

Recombinant human granulocyte colony stimulating factor (rhG-CSF), a therapeutic protein drug and hematopoietic growth factor, is one of the most exciting new drugs developed in the past 20 years and one of the five best-selling genetic drugs in the world (Metcalf 1988; Walsh 2010). The technology to produce rhG-CSF is conventionally divided into two phases, refolding and subsequent purification; however, this technology has low refolding yields and high production costs. If the refolding and purification of rhG-CSF can be combined into a one-step operation, the production cost will be greatly reduced. Unfortunately, unfolded rhG-CSF in U state is strongly hydrophobic and contains five cysteine residues that form two intramolecular disulphide bonds (Cys36-Cys42 and Cys64-Cys74), leaving one cysteine residue free at position and forming incorrect disulphide bonds. Another problem is that the inclusion body extract of the protein in high concentrations of urea or GdnHCl solution immediately precipitates upon contact with an aqueous solution in absence of denaturant, which creates a very difficult problem for both the various conventional refolding methods and PFLC. Our group (Wang et al. 2006, 2007, 2008a, 2009a; Wang and Geng 2012) chose rhG-CSF as a target protein in an attempt to systematically solve these problems using the previously described four types of PFLC (PF-HIC, PF-SEC, PF-IEC, and PF-AFC). The validation of protein refolding by each of the four PFLC types and their efficiencies are shown in Table 3.

As shown in Table 3, all four of the commonly used PFLC methods can be employed for the simultaneous refolding and purification of rhG-CSF in a single step in 40 min. With the exception of PF-SEC, these PFLC methods can achieve greater than 95 % purity and specific activities of greater than 1×10^8 IU/mg. However, Table 3 also indicates that the mass recovery is very low, with the highest recovery of only 49 %. This outcome

Table 2 Effect of GSH/GSSH (3:1) on bioactivity recovery of reduced/denatured RNase A

t/h	Recovery of bioactivity (%)			*t*/h	Recovery of bioactivity (%)		
	IEC column	GSH/GSSG column[a]	GSH/GSSG column[b]		IEC column	GSH/GSSG column[a]	GSH/GSSG column[b]
0	7.3 ± 3.0	25.6 ± 2.2	23.5 ± 3.8	4	64.7 ± 1.2	75.7 ± 2.8	74.8 ± 1.3
1	13.1 ± 2.3	43.1 ± 3.4	41.5 ± 2.5	5	68.3 ± 0.8	77.3 ± 3.2	76.2 ± 1.6
2	31.4 ± 2.9	61.4 ± 1.5	58.3 ± 2.1	6	71.5 ± 3.6	79.5 ± 3.4	78.6 ± 1.8
3	50.7 ± 2.4	70.7 ± 1.1	69.5 ± 3.2	7	73.4 ± 4.0	82.4 ± 2.0	81.5 ± 4.3

[a] With GSH/GSSG added to mobile phase

[b] With GSH/GSSG added to sample collection (Ke and Meng 2012)

Table 3 Comparison of PFLC refolding for rhG-CSF

Refolding methods	Media and column size	Additives	Mass recovery (%)	Purity (%)	Bioactivity ($\times 10^8$ IU/mg)	References
IEC	Q Sepharose Fast Flow 200 mm × 12 mm i.d.	3.0 mol/L urea, 2.5 m mol/L GSH, 0.8 m mol/L GSSG	49.0	96	3.0	Wang et al. (2007)
SEC	Superdex 75 200 mm × 26 mm i.d.	15 % glycerol (v/v), 2.5 m mol/L GSH, 0.8 m mol/L GSSG	30.0	83	1.2	Wang et al. (2006)
Urea gradient SEC	Superdex 75 200 mm × 16 mm i.d.	15 % glycerol (v/v), 2.5 m mol/L GSH, 0.8 m mol/L GSSG	46.1	NR	1.0	Wang et al. (2008a)
AFC	Cu(II)-iminodiacetic acid (IDA)-IMAC 100 mm × 12 mm i.d.	2.0 mol/L urea	39.0	97	2.3	Wang et al. (2009a)
HIC	NR 10 mm × 200 mm i.d.	NR	36.9	95.4	2.3	Wang et al. (2012)

NR not reported

could be attributed to either strong hydrophobicity of the protein in U state or incomplete retardation of the U state rhG-CSF on the top of the columns. Although a new method was used to solubilise the rhG-CSF inclusion bodies to improve the mass recovery, the effect of this method was insignificant (Dasari et al. 2008; Wang et al. 2009b). Our group has recently synthesised a new arginine-type chromatographic stationary phase to perform refolding and purification of rhG-CSF expressed in *E. coli* that can increase the mass recovery to 68 % (Zhou 2009). Solubilisation of inclusion bodies by high-pH solution and protein refolding using liquid chromatography were combined and successfully applied to rhG-CSF expressed in *E. coli*, which caused a significant increase in the mass recovery to 73 % (Li et al. 2012a, b).

PFLC with mixed-mode stationary phase

Due to the basically identical operation of PFLC and basic LC, as long as the denatured protein samples are injected onto an appropriate column and eluted out, these denatured protein molecules should refold or renature during the chromatographic process. In the early stages of PFLC, denatured proteins were often thought to be simply adsorbed onto the column to prevent the association and/or precipitation of proteins in U state (Liu et al. 2007). Over the years, it has been shown that this is simply not the case. The popular use of the aforementioned four types of PFLC, which have various renaturation efficiencies, is powerful evidence. For a specific type of PFLC, any improvement of stationary phase and new understanding of protein retention mechanisms can be used to develop PFLC.

However, one significant difference between LC and PFLC is that for the former, the target protein has the same N state in the sample solution and the eluted solution, while for the latter, the target protein in the sample solution is in U state. According to the conventional renaturation condition, the target protein is required to become N state by PFLC, which may not meet the sampling condition that the target protein should be in U state and must be completely retarded on the top of the column. For example, the isoelectric point of a native protein is 5.0, so refolding should occur around pH 5.0 using a PF-IEC column. However, the same protein in its U state may be partially retarded at pH 5.0, while the remaining protein may be retarded at pH 8.0.

Thus, as the pH of the employed mobile phase (according to pH 5.0) is selected, the latter fraction would directly pass through the column and elute with the solvent peak. On the other hand, the two fractions of the protein could be retarded at the top of the column; one fraction may be completely refolded, but the other may be tightly and seemingly irreversibly adsorbed by the same stationary phase. An ideal stationary phase should overcome these two problems (Gao et al. 2008).

The development of MMC provides an additional option for performing this request. The selection of stationary and mobile phases accompanying experimental PFLC conditions contributes to the protein folding efficiency. MMC is a chromatographic method in which multiple interaction modes occur between the stationary phase and the solutes in the feed (Geng et al. 2009; Liu et al. 2009a; Yang and Geng 2011; Lu et al. 2013). MMC has high selectivity and a remarkably high loading capacity. If the MMC method is employed, the target protein in the U state can be adsorbed and refolded via two or more interaction forces. The recovery of mass and bioactivity will be greatly increased (Kimberly et al. 2010). Refolding with simultaneous purification of hDll1-RGD was studied using a kind of Capto mixed-mode weak cation-exchange chromatography. The results indicated that the mass recovery of the hDll1-RGD was 43.6 % and the purity was 98.0 % (Wu 2013).

The most popular MMC for protein separation has been recognised to be IEC and HIC mixed-mode because these approaches have better orthogonality. The special ligand of the stationary phase combines two or more interaction modes (dispersion, hydrogen bonding, and electrostatic interactions). The ratio of dispersion to hydrogen bonding can adjust the strength of the hydrophobic interaction. Studies have shown that MMC stationary phases that simultaneously contain multiple-functions have very good application prospects in the separation of natural biological macromolecules (Kennedy et al. 1986). However, PF-IEC and PF-HIC have some drawbacks; for example, IEC has low ionic strength and HIC has ionic strength that is too high, which is problematic for buffer exchange as the target protein transfers from one mode to the other mode.

A character of MMC is that the orthogonal retention is performed with the same MMC stationary phase, but two different characters of mobile phases (Mehander et al. 1989; Tong et al. 2013). A stationary phase of MMC (IEC, HIC) for performing PF-IEC can be created using a gradient from low to high concentration of salt, while for PF-HIC; the gradient may be created from high to low concentration of salt, although the same two solutions of different concentrations can be employed for the two mobile phases in the two modes. As a result, the optimisation of conditions for PFLC is not only more flexible, but also greatly simplifies the PFLC process.

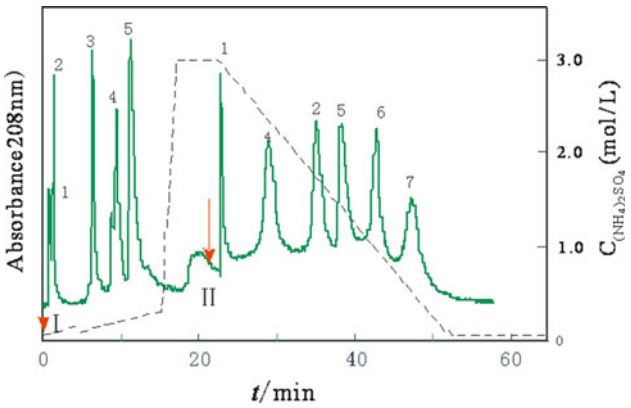

Fig. 2 Chromatogram of standard proteins separated with second collected fractions by on-line IEC-HIC. Conditions: mobile phase A: 3.0 M $(NH_4)_2SO_4$, 20.0 mM KH_2PO_4, pH 6.5; mobile phase B: 20.0 mM KH_2PO_4, pH 6.5; flow rate: 1.0 mL/min; and detection: 280 nm. The non-linear gradient elution was as follows: 0.0–15.0 min, 100–90 % B; 5.0–17.5 min, 90 % B–100 % A; 17.5–22.5 min, 100 % A; and 22.5–52.5 min, 100–100 % B. The gradient elution programme is represented by the *dashed line* in the chromatogram. The second fractions were collected at 22 min. Peaks: *1* solvent peak, *2* Myo, *3* RNase A, *4* Cyt c, *5* Lys, *6* α-Amy, and *7* Ins

Two-dimensional chromatographic column in MMC has been reported for performing off-line 2D-LC-1C for the fast separation of active proteins. Two-dimensional column (2D column) has been defined as an efficient column with two separation modes that is superior or at least comparable to the two corresponding conventional columns (Geng et al. 2009; Wang et al. 2012; Jia et al. 2012). Thus, the 2D column can replace two single-mode columns using off-line approaches in all cases.

As shown in Fig. 2, a 2D column with weak cation exchange (WCX) and HIC separation modes can replace a commercial WCX column and commercial HIC column for protein separation (shown on the left side and right side of the perpendicular arrow). The two separation modes can simultaneously perform adsorption during sampling and refolding during gradient elution without changing the 2D column and the mobile phase, but changing the order of the weak and strong solutions.

Yang et al. (2013) have employed the 2D column for on-line fast separation and purification of cytochrome C (Cyt C) from bovine pancreas. The procedure was performed in a closed system with positive pressure in 52 min. The purity and mass recovery of the ultimately obtained Cyt C were determined to be 94.7 and 80.5 %, respectively. Although this method seems not to have involved PFLC, the operation protocol is the same as that for on-line fast 2D-PFLC using a single 2D column. Jia et al. (2012) found that the width of the "U-shape" curve was experimentally found to relate to the chromatographically dynamic factor, while the magnitude of the critical point of the "U-shape" curve relates to the protein retention.

Bai and Geng (2011) have employed a 2D (HIC, WCX) column for off-line refolding with simultaneous separation of guanidine-denatured lysozyme and ribonuclease A. Using the same 2D column and two mobile phases, the two enzymes were renatured and separated in the WCX and HIC modes in 25 min. The mass recovery of the renatured lysozyme and ribonuclease A in the HIC mode surpassed 85 %. One type of hydrophobic stationary phase with electronic interaction was developed by bonding β-phenylethylamine as the functional ligand to the silica, which can allow renaturation and simultaneous purification of the recombinant human Flt3 ligand (rhFL) (Jia 2010). The covalent bonding of glutathione/glutathione (GSH/GSSG) to the surface of a silica-based stationary phase has also recently been reported for off-line protein folding (Ke and Meng 2012). The glutathione-bonded stationary phase was tested in the WCX and HIC separation mode. Using a protein concentration of 5 mg/mL flow rate of 0.2 mL/min and no GSH/GSSG in the mobile phase, the bioactivity recovery of denatured RNase A reached 39.5 ± 3.8 % when refolded using the glutathione-bonded column, compared to nearly 0 % using a common IEC column. The column promoted the correct refolding of denatured protein disulphide bonds. As shown in Table 2, after RNase A was refolded by the association of electrostatic and hydrophobic interaction forces and GSH/GSSG was added to the fraction containing RNAse A and left to stand for several hours, the bioactivity recovery reached 81.5 ± 4.3 %.

Four commercial columns of both weak anion-exchange chromatography and WCX have been found to have a mixed-mode character (both IEC and HIC) (Sun et al. 2010). Thus, all protein folding by IEC can be considered for refolding by two types of MMC. The retention mechanism of proteins on an MMC stationary phase has been reported as the "molecular orientation of protein-stoichiometric displacement theory (SDT)" (Liu et al. 2009a). The mechanism of protein refolding by MMC is quite complicated. An HIC stationary phase was prepared by phenylethylamine, which has a hydrophobic group (phenyl) and a charged group (amino) ligand. This stationary phase was dominated by hydrophobic interaction and simultaneously supplemented by weak cation-exchange interaction. The bifunctional stationary phase was successfully employed for the renaturation and simultaneous purification of denatured lysozyme (Gao et al. 2013).

PFLC in molecular biology

One of the advantages of PFLC is the ability to control protein folding. The character, preparation, dynamics, existing intermediates, equilibrium between intermediates and native state, and changes in molecular conformation of proteins have been reported, as a protein in U state folds into its corresponding N state (Hou et al. 2010). Refolding can be performed using either PFLC alone or in combination with other methods. The five types of PFLC can be employed based on the character of the existing intermediates (such as hydrophobic in PF-HIC, electrostatic distribution in PF-IEC, sizes in PF-SEC, and affinity in PF-AFC, plusing PF-RPLC in the circumstance) and the changes in molecular conformation. Protein identification combined with other methods (such as MS) by PF-RPLC. A PF-RPLC was employed to investigate the structural changes that occur during a biomolecular self-assembly process.

The urea-denatured α-amylase (α-Amy) has been reported to simultaneously denature and separate from its stable intermediates (Bai et al. 1997). Recently, α-Chy has been considered to be a typical protein in multiple investigations (Ke et al. 2013). PF-RPLC has been employed to investigate the structural changes that occur during a biomolecular self-assembly process. The temporal development of protein structure during S100A11 folding and dimerisation was probed by oxidative labelling and LC–mass spectrometry (LC–MS) (Bradley et al. 2011). First, IEC has recently been described in detail as a typical example for separating the native form and a partially structured intermediate of the folding of the amyloidogenic protein beta2-microglobulin (Bertoietti et al. 2013). Using a strong anion-exchange column that accounts for the differences in charge exposure of the two conformers, a LC–UV method was initially optimised in terms of mobile phase pH, composition and temperature. The preferred mobile phase conditions that afforded useful information were 35 mM ammonium formate, pH 7.4 at 25 °C. The dynamic equilibrium of the two species was demonstrated upon increasing the concentration of acetonitrile in the protein sample. Then, the chromatographic method was transferred to MS detection, and the respective charge state distributions of the separated conformers were identified. The LC–MS results demonstrated that one of the conformers was partially unfolded, compared with the native and more compact species. The concordance with previous results obtained in free solution by capillary electrophoresis suggests that strong ion exchange LC–MS does not alter beta2-microglobulin conformation and maintains the dynamic equilibrium already observed between the native protein and its folding intermediate.

SDT was employed to investigate the changes in the interaction forces in various denaturants and to distinguish the N state of a protein from its unfolding M state by PF-HIC (Ke et al. 2009). The two linear parameters log$I$ and Z (for their physical meanings, see later) of the SDT-R), log$I$ (a constant relating to the affinity of 1 mol of the protein for the stationary phase), and j (the affinity of water to the stationary

phase of HIC) which is obtained from the slope of the linear plot of log$I$ vs Z) were widely employed for characterising the changes in molecular conformation of proteins and molecular structure of small solutes (Valko et al. 1993) and biopolymers (Lin and Karger 1990; Kennedy et al. 1986; Geng et al. 1990). Protein molecules are more extensively in the N state with normal molecular conformations than they in an U, also M state with abnormal protein folding molecular conformations. The M and N states of a protein must have a different magnitude of log$I$ and Z.

A protein in N state has a correct tertiary- or quaternary structure, in comparison to protein tight contacting to stationary phase of HIC, it allows flexible changes in molecular conformation in buffers, and the hydrophobic amino acid residues exist inside the protein molecules. However, proteins in the M states are compact which do not present flexible changes in molecular conformation and U states are random coil without a fixed conformation. In protein in U state, the hydrophobic amino acid residues are exposed on the surface of the protein molecules and protein in M states is generally modelled as having undergone a hydrophobic collapse. For the latter case, both hydrophobic and hydrophilic amino acid residues of protein will contact to the stationary surface. In this circumstance, the interaction between protein and stationary phase is selective.

The parameter j can be employed to distinguish selective to non-selective interactions between protein and stationary phase of HIC. A simple way is to do plot of log$I$ vs Z. An excellent linearity indicates the interaction to be non-selective, i.e. otherwise, it to be selective one. In short words, when the change in Z value is large, the structure of the protein changes to a large extent, such as when a protein in the U state converts to an N state. In contrast, proteins in the U and/or M states cannot be refolded by HIC, so their molecular structures and corresponding characterisation parameters will not change significantly (if these characterised parameters can be identified and exactly measured).

Acknowledgments This work was supported by the Foundation of Key Laboratory of Modern Separation Science in Shaanxi Province (Nos. 2010JS104; 11JS098), the Foundation of Science and Technology in Shaanxi Province (No. 2010K12-01-05), and the foundation of Provincial Key Description of Analytical Chemistry of Shaanxi Subject (ZDXKL00402).

Conflict of interest The authors declare that they have no conflict of interest.

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